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EXPERIMENTS WITH THE WINOGRADSKY SPONTANEOUS CULTURE TEST ON IOWA SOILS

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Winogradsky (1)[1] in 1925 developed the spontaneous culture method for studying the occurrence and activities of the aerobic non-symbiotic nitrogen fixing organisms in the soil. Using this method in conjunction with the silica gel method (2, 3) he was able to determine the nitrogen fixing power of the soil and the relative number of soil micro-organisms which will grow on the surface of the spontaneous culture plates. These organisms Winogradsky believed represented the aerobic non-symbiotic nitrogen fixing organisms of the soil and members of the Azotobacter genus were very prevalent in the colonies which developed.

Several workers have used the spontaneous culture method as a test for the presence of Azotobacter in soils, and for detecting plant food deficiencies. The procedure followed in recent work is essentially as follows: a carbohydrate energy material, generally starch or sucrose, is added to the soil to stimulate the growth of the aerobic organisms which develop on the surface of the plates. The treatment is varied by adding lime, phosphorus and potassium in varying amounts separately and in combination.

Some previous tests of the method have been made in Iowa and the results were not entirely satisfactory. Further work has been conducted recently in the attempt to solve some of the difficulties encountered and to ascertain whether or not the test may be modified so that it can be used for determining the plant food deficiencies of Iowa soils. No attempt is made here to discuss all the data contained in the tables, but merely to point out some facts indicated by the work.

EXPERIMENTAL

Several series of plots located on Carrington loam at the Agronomy Farm of the Iowa Agricultural Experiment Station were chosen for this study as records of previous treatments and crop yields on each of the plots are available. In addition, a sample of Colorado soil was secured through the courtesy of Mr. Robert Gardner of Rocky Ford, Colorado Experiment Station, and a

sample of Utah soil was sent in by Dr. T. L. Martin of the Brigham Young University at Provo, Utah.

The samples were dried sufficiently to pass a 20-mesh sieve as soon as they were brought into the laboratory. After sieving they were placed in Mason jars and samples for the later tests were taken from these jars. pH determinations made by the quinhydrone electrode method show that the soils ranged from pH 5.89 to pH 8.45, all practically within the generally accepted range for Azotobacter growth.

The procedure for making up the spontaneous culture plates was as follows: three 50-gram samples of each soil were weighed out and treated as follows: (a) potato starch 5 per cent, check; (b) starch and 0.4 per cent lime; and (c) starch and lime and 0.6 per cent secondary sodium phosphate.

After the materials were thoroughly mixed with the soil, sufficient water was added to make a thick paste. The moistened soil was then packed into duplicate halves of petri dishes and the surface smoothed by means of a moist spatula. The plates were then placed in moist chamber dishes and incubated at 28 degrees Centigrade.

In some of the later experiments mannitol was used as energy material to replace the starch as it was found that the mannitol stimulated a more rapid and vigorous growth.

Preliminary experiments on the Carrington loam from plots 907 and 909 indicated that it was possible to obtain the typical Azotobacter growth, described by Winogradsky (1), on the soil from plot 907 when starch, lime and phosphate were added but there was no growth when the phosphate was omitted from the treatment. With the soil from plot 909 no growth was obtained with any of the treatments. Table I gives the field treatments of all the Iowa soils used in the experiment.

A comparison of the growth on the spontaneous cultures from humid and arid soils is shown in table II. It is of particular interest to note the development of the typical Azotobacter growth with the arid soil. With the Colorado soil small slow growing colonies were produced in 72 hours when starch or starch and lime were added but when starch, lime and phosphate were supplied, vigorous, rapid growing colonies developed in 72 hours. Neither the 1 to 1 nor the 3 to 1 mixtures of Carrington loam from plot 909 and the Colorado soil changed the character or amount of growth common to the Colorado soil. Similar results were obtained in the 1 to 1 and the 3 to 1 mixtures of the Carrington loam from plot 909 and the Utah soil. No influence was shown on the character or amount of

Table I — Carrington Loam Soils of Iowa Used in Experimental Work

Plot No.	Treatment
700	Green manure (clover) and lime in sufficient amount to neutralize the acidity as indicated by lime requirement tests once in four years.
701	Same as 700 plus 500 pounds of raw rock phosphate every four years.
702	Same as 700 plus 1000 pounds of raw rock phosphate every four years.
703	Same as 700 plus 1500 pounds of raw rock phosphate every four years.
704	Same as 700 plus 2000 pounds of raw rock phosphate every four years.
705	Same as 700.
907	Farm manure and lime. Manure applied at rate of 8 tons per acre, once every four years.
908	Lime applied as required to neutralize acidity as indicated by lime requirement tests, once every four years.
909	Check — No treament.
1000	Check — No treament.
1001	Manure, once every four years at the rate of 8 tons per acre.
1002	Manure as in 1001 plus limestone sufficient to neutralize acidity as shown by lime requirement tests, once in four years.
1003	Manure and lime as in 1002 plus raw rock phosphate at the rate of 2000 pounds per acre once in four years.
1004	Manure and lime as in 1002 plus superphosphate at the rate of 150 pounds per acre every year.
1005	Check — No treatment.

growth, common to the Utah soil, by the addition of a large amount of the Carrington loam which failed to support a spontaneous culture growth under similar treatment. It may also be of interest to note that the typical Azotobacter growth appeared 24 hours earlier on the plates of Colorado soil than on the plates made from the Utah soil.

Attempts to secure the typical Azotobacter growth on Carrington loam from plots 909 and 907 by inoculation with pure and mixed cultures are summarized in tables III and IV. Inoculation, as shown in table III, did not in any case bring about a typical Azotobacter growth on the soil from plot 909. On the soil from plot 907 where a typical, but not vigorous, Azotobacter growth appeared at 72 hours when starch, lime and phosphate were used in the test, all inoculation treatments inhibited the development of the normal soil

Table II — A comparison of the development of spontaneous cultures on Carrington loam of Iowa and Colorado and Utah soils, after incubation at 28 degrees Centigrade

Soil	Check Plates (starch only)	Plates Receiving Starch and Lime	Plates Receiving Starch, Lime and Phosphate
Carrington 909	No growth	No growth	No growth
Colorado	Small, slow growing colonies in 72 hours	Small, slow growing colonies in 72 hours	Typical, vigorous growing colonies in 72 hours
Utah	Typical colonies in 96 hours	Typical colonies in 96 hours	Typical colonies in 96 hours
½ Carrington 909 ½ Colorado	Small, slow growing colonies in 72 hours	Small, slow growing colonies in 72 hours	Typical, vigorous growing colonies in 72 hours
½ Carrington 909 ½ Utah	Typical colonies in 96 hours	Typical colonies in 96 hours	Typical colonies in 96 hours
¾ Carrington 909 ¼ Colorado	Small, slow growing colonies in 72 hours	Small, slow growing colonies in 72 hours	Small, slow growing colonies in 72 hours
¾ Carrington 909 ¼ Utah	Typical colonies in 96 hours	Typical colonies in 96 hours	Typical colonies in 96 hours

flora which gave the typical Azotobacter growth. This is difficult to explain. However, it appears from the results given in table IV that the one cubic centimeter inoculum of the original suspension of *Azotobacter chroococcum* was too large when smaller amounts of inoculum were used on the plates of soil for plot 907, with starch as energy material, no inhibition of normal Azotobacter growth occurred.

It also appears from Table IV that the energy source is very important for the development of the typical Azotobacter colonies on the spontaneous culture plates. It is evident that a much more vigorous and rapid growing culture is produced by the use of mannitol as energy material than where starch is used. The effect of the varying amounts of inoculum is shown with the larger amounts of mannitol added.

It seemed advisable to study the test further on soils from differently treated plots on the Agronomy Farm to determine whether or not it would give indications of previous phosphorus additions to the soil. Mannitol was used as energy material as it gave a more vigorous and rapid growing culture in previous tests and made the test more sensitive. On the spontaneous culture plates of the soil from every plot listed in table V the typical growth

Table III — Appearance of typical colony growth after inoculation of soil with various pure and mixed cultures of organisms

Soil	Treatments					
	Starch Lime Phosphate	Starch Lime Phosphate *Azotobacter chroococcum	Starch Lime Phosphate *Azotobacter beijerinckii	Starch Lime Phosphate *Azotobacter vinelandii	Starch Lime Phosphate **Organisms from Colo. Soil	Starch Lime Phosphate **Organisms from Utah Soil
Carrington 909	No growth in 120 hours	No growth in 120 hours	No growth in 120 hours	No growth in 120 hours	No growth in 120 hours	No growth in 120 hours
Carrington 909	Typical growth in 72 hours	No growth in 72 hours	No growth in 72 hours	No growth in 72 hours	No growth in 72 hours	No growth in 72 hours

* Suspension made from a 10 cc. suspension of a 48 hour slant of pure culture.

** Suspension made from colonies which developed on the surface of spontaneous culture plates of the soil. Organisms removed from the surface of the soil plate with a sterile needle and placed in physiological salt solution until a heavy suspension of the organisms was obtained. In each case one cubic centimeter of the suspension was used to inoculate 50 grams of soil.

Table IV — Effects of size of inoculum, and various amounts of mannitol compared with starch as energy material, upon the amount of growth and time of appearance of spontaneous cultures on Carrington loam soil from plot 907 (all observations made at 48 hours except where otherwise noted in table)*

TREATMENT	NO INOCULATION	1 CC. OF ORIGINAL SUSPENSION	0.1 CC. OF ORIGINAL SUSPENSION	0.01 CC. OF ORIGINAL SUSPENSION	0.001 CC. OF ORIGINAL SUSPENSION
2.5 per cent mannitol + lime + phosphate	++**	++++	++++	++	+(96 hrs.)
1.25 per cent mannitol + lime + phosphate	++	++++	+++	+++	+++(72 hrs.)
0.625 per cent mannitol + lime + phosphate	++	++	++	++	++(72 hrs.)
0.312 per cent mannitol + lime + phosphate	+	+	+	+	++(72 hrs.)
5.0 per cent starch + lime + phosphate	+	—	+	+	+(96 hrs.)
	(96 hrs.)	(120 hrs.)		(96 hrs.)	

* Inoculum made from a 10 cc. suspension of a 48 hour slant of a pure culture of *Azotobacter chroococcum.*
** + designates a scant but noticeable growth.
++ designates an average growth.
+++ designates a heavy vigorous growth where it is difficult to distinguish the colonies from each other.
++++ designates a very dense growth, overgrowing the entire plate so that the individual colonies are not distinguishable.

appeared only where the treatment included mannitol, lime and a phosphate, indicating that the soil from all the plots needed additional applications of phosphate. Crop yield records on the plots 700 to 705, however, show that no added crop yields were obtained with applications of raw rock phosphate greater than 1000 pounds per acre every four years. Some other factor than phosphorus has evidently become the limiting factor in crop production in this case. Yet this test would be interpreted to indicate the need of additional applications of a phosphate to the soils of these plots.

It is evident that the phosphorus in those soils which have received the larger applications of raw rock phosphate was not in a form available to the organisms developing on the spontaneous culture plates inasmuch as there was no development without the

Table V — The development and time of appearance of spontaneous cultures on plates of soil from different experimental plots (see Table I for treatments of plots listed below)

Plot No.	Check Plates (Mannitol* Only)	Plates Receiving Mannitol + Lime	Plates Receiving Mannitol + Lime + Phosphate
908	No growth	No growth	Average growth in 48 hours
1000	"	"	Scant growth in 120 hours
1001	"	"	Scant growth in 48 hours
1002	"	"	Scant growth in 48 hours
1003	"	"	Scant growth in 48 hours
1004	"	"	Scant growth in 96 hours
1005	"	"	Very scant growth in 120 hrs.
700	"	"	Average growth in 48 hours
701	"	"	Average growth in 48 hours
702	"	"	Average growth in 48 hours
703	"	"	Average growth in 48 hours
704	"	"	Average growth in 48 hours
705	"	"	Average growth in 48 hours

* Mannitol was substituted for starch, using 0.625 per cent.

Table VI — Effect of changing the phosphorus source on the development of the spontaneous cultures on plates of Carrington loam from plot 704

Treatment	Observations
0.625 per cent mannitol, lime and 0.6 per cent secondary sodium phosphate	A heavy vigorous growth, with the individual colonies difficultly distinguishable appeared after 72 hours incubation
0.625 per cent mannitol, lime and 0.6 per cent raw rock phosphate	No growth appeared after 120 hours incubation
0.625 per cent mannitol, lime and 0.6 per cent superphosphate	A very scant growth of three or four small colonies appeared after 72 hours incubation.

addition of phosphorus in a readily available form. The results in table VI indicate that neither the raw rock phosphate nor the superphosphate is suitable as a phosphorus source for the development of the organisms which appear on the spontaneous culture plates. The superphosphate seemed to be only very slightly available as few colonies developed on the plates made from soils which had received superphosphate.

SUMMARY AND CONCLUSIONS

Some factor or group of factors appears to be operative in the soils used in this experiment, preventing a satisfactory use of the spontaneous culture method for determining their plant food deficiencies. This factor is not sufficiently active, however, to prevent the normal development of the typical Azotobacter growth of the arid soils of Colorado and Utah when the Iowa soil is mixed in a 3 to 1 proportion with either of them. It seems hardly possible that the soils used in this experiment lack the organisms which develop on the spontaneous culture plates for with proper treatments in the tests the typical growth appears on the majority of the spontaneous culture plates made from the soils of the different plots. Of course, it is possible that some soils with a lower pH than these tested would not support the soil flora on which the test depends.

The marked influence, on the character and amount of growth of the organisms appearing on the spontaneous culture plates, brought about by the change from starch to mannitol as an energy source, indicates a lack of sensitiveness of the test, probably due to an unsatisfactory balance among the factors influencing the growth of the organisms. Possibly an optimum balance is more nearly attained in the arid soils than in the Iowa soils tested which would explain the more reliable results obtained by use of the test for the western soils.

In the light of the experiments set forth in this paper it seems that the Winogradsky spontaneous culture test as heretofore employed is not entirely suited for the accurate determination of plant food deficiencies in Iowa soils. However, the authors feel that it may be possible to modify the method so that the plant food deficiencies of these soils may be determined with reasonable accuracy by this test. Work on the method is in progress to determine, if possible, the modifications necessary to make the test applicable to the soils of Iowa.

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